

THERMOPHILIC FUNGI ASSOCIATED WITH THE CULTIVATION OF *AGARICUS BISPORUS* (LANGE) SINGER

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ABSTRACT

Thermophilic fungi were isolated from compost used in experimental mushroom culture. Three methods, the moist chamber, direct plating and dilution plate methods were used at intervals during the process of cultivation to study the occurrence and succession of these specialized fungi up to the stage of the formation of the first sporophores.

Ten species of thermophilic moulds were isolated. They were *Aspergillus fumigatus*, *Chaetomium thermophile* var. *dissitum*, *Humicola grisea* var. *thermoidea*, *Humicola insolens*, *Humicola lanuginosa*, *Malbranchea pulchella* var. *sulfurea*, *Mucor miehei*, *M. pusillus*, *Talaromyces duponti* and *Torula thermophila*. The frequency of occurrence of six dominant thermophiles is considered in detail and correlated with the variation in compost temperature.

UITTREKSEL

TERMOFIELE FUNGI GEASSOSIEER MET DIE KWEEK VAN *AGARICUS BISPORUS* (LANGE) SINGER

Termofiele fungi is uit kompos van 'n eksperimentele sampioenwekery geïsoleer. Drie metodes nl. vogtige kamer, direkte uitplating en verdunningsplate is gebruik om die voorkoms en suksessiepatrone van hierdie gespesialiseerde fungi tydens die hele proses van sampioenweking tot en met die verskyning van die eerste vrugliggame na te gaan. Tien termofiele fungi is geïsoleer. Hulle is *Aspergillus fumigatus*, *Chaetomium thermophile* var. *dissitum*, *Humicola grisea* var. *thermoidea*, *Humicola insolens*, *Humicola lanuginosa*, *Malbranchea pulchella* var. *sulfurea*, *Mucor miehei*, *M. pusillus*, *Talaromyces duponti* en *Torula thermophila*. Die frekwensie van voorkoms van ses dominante termofiele word in besonderhede bespreek en in verband gebring met die variasies in komposttemperatuur.

INTRODUCTION

During a study of thermophilic fungi occurring in South Africa, attention was turned to composting substrates used in the commercial cultivation of the mushroom *Agaricus bisporus* (Lange) Singer. The mushroom is grown in a mixture composed largely of wheat straw and horse manure. Before *A. bisporus* will grow well in this substrate it must undergo a certain amount of deterioration known as composting. The commercial preparation of mushroom compost in-

volves two phases. Phase I is essentially a fermentation process resulting in the release of energy in the form of heat. This thermogenesis is favoured by aerobic conditions which are maintained by restricting the width and height of the compost piles and by repeated turning of the composting material. Internal pile temperatures may reach 80 °C or more during this stage. Phase II takes place in controlled temperature rooms after wooden trays have been filled with the compost. During this phase, known as "peak heating", the temperature within the compost is maintained between 50 and 60 °C by aeration of the room. The air temperature of the room is held in the range of 35–45 °C. It is now known that thermogenesis by micro-organisms initiates, at least the heating of Phase I and produces the heat in Phase II (Fergus, 1964).

Many micro-organisms associated with the composting process have been isolated and these include both mesophilic and thermophilic bacteria, actinomycetes and fungi (Bels-Koning, *et al.*, 1962; Hensen, 1957; Pope, Knaust & Knaust, 1962; Reese, 1946; Renoux-Blondeau, 1959 and Tendler & Burkholder, 1961). The first detailed study of thermophilic bacteria and fungi active in compost at temperatures in excess of 50 °C was that of Waksman, *et al.* (1939). More recent work has indicated the importance of thermophilic fungi in mushroom cultivation. Bels-Koning, *et al.* (1962) showed that the thermophilic species of *Humicola* are important in the composting process. The thermophilic and thermotolerant fungi and actinomycetes colonizing compost during peak heat were studied by Fergus (1964) while Cooney and Emerson (1964) and Fergus and Sinden (1969) described some thermotolerant fungi from mushroom compost. Chang and Hudson (1967) made an extensive study of the ecology of wheat straw compost and they isolated a number of thermophilic fungi. In a study of the microbial changes taking place in composting wheat straw/horse manure mixture Hayes (1968) found that thermophilic fungi belonging to the genera *Humicola*, *Mucor*, *Stilbella* and *Torula* were active at different stages in the composting process. Fordyce (1970) determined that many of the thermophilic fungi active in Phase I and Phase II of composting were also present in the compost during mushroom spawn development and sporophore production but that these fungi, particularly species of *Chaetomium*, *Humicola* and *Torula*, showed a definite decline in number as time progressed. Staněk (1972) concentrated on the period of peak fermentation in his study of thermophilic micro-organisms colonizing mushroom compost.

The aim of this study is to contribute to the present data on the importance of thermophilic fungi colonizing mushroom compost during the entire period, from the start of Phase I until the stage of the appearance of the first fruiting bodies. An attempt was also made to study the succession of these fungi in the compost and to determine their frequency of occurrence as the composting, spawn run (colonization of compost by mushroom mycelium) and sporophore development progressed. In order to do this a pilot plant was set up at the University of Pretoria where the whole process could be executed under strictly controlled conditions.

MATERIAL AND METHODS

Pilot mushroom cultivation

The approximate proportions of the ingredients that were used for the compost which was to be analysed microbiologically are shown in Table 1. The materials were thoroughly mixed and watered to bring the moisture content to about 78 %. On day 0, at the start of Phase I composting, the materials were turned and mixed and stacked with the aid of a wire netting frame, 1×1×1 metre, under an open shelter. This small experimental stack was subsequently turned and mixed on days 3, 6 and 9 and watered when necessary. On day 10, at the end of Phase I, the compost transferred to wooden trays (internal dimensions 800×1400×200 mm) and moved into a room in a phytotron for Phase II composting. As a ready supply of steam was not available the air temperature was raised by means of electrical heaters to 40 °C while a high relative air humidity was maintained with the aid of a motorized humidifier. At the end of this stage the compost was allowed to cool down after which it was spawned at a concentration of 400 g grain spawn per square metre of bed area. The variety of mushroom used was the "pure white" A32 commonly grown commercially in South Africa.

TABLE 1.

Relative proportions (approximate volume) of ingredients used in preparing stack composts.

Substrate	Proportion of ingredients
Wheat straw	50
Horse manure (stable bedding)	35
Chicken manure	10
Gypsum (hydrated calcium sulphate)	4
Superphosphate	1

The spawn was allowed to colonize the compost over a period of 10 days at a room temperature of 25 °C and a relative air humidity of 95 %. The compost was then "cased", i.e. it was covered with a layer of casing soil to a depth of 25–35 mm. Casing soil consists of equal parts of ground peat and limestone. The cased trays were moved to the growth room in a phytotron where they were incubated in darkness at a temperature of 18 °C and a relative air humidity of 85 % up till the stage that the first flush of sporophores appeared (Fig. 1), usually around day 46.

Temperature fluctuations in the compost, especially during Phases I and II, were measured by inserting a long thermometer vertically into the centre of the stack to a depth of 400 mm in the case of Phase I and to a depth of 100 mm in the Phase II compost. Readings were taken daily (Table 2). The pH was measured regularly using a Beckman pH meter with a glass electrode. About 25 g compost was soaked in 100 ml of distilled water (pH 5.3) for 1 hour prior to pH determinations (Table 2).

Sampling

Previous investigators have pointed out the problems associated with sampling of composting materials (Lambert, 1941; Hayes, 1968). To minimise errors which could arise from sampling from different zones in the stack during Phase I, all samples were composite samples composed of material taken at regular depth intervals from the surface of the stack to a point 460 mm below the surface at the centre of the compost stack. This area is considered by Lambert (1941) to be microbiologically the most active. Samples taken during Phase II and later stages of cultivation were mixed subsamples of compost from all regions of the compost.

Thermophilic fungi

Three methods were employed to study the thermophilic fungal flora and to follow the patterns of succession. In one method compost pieces were cut or broken into small pieces and placed in sterile large Petri dishes that had previously been lined with several layers of paper towelling moistened with distilled water and autoclaved. These moist chambers containing the organic substrates were

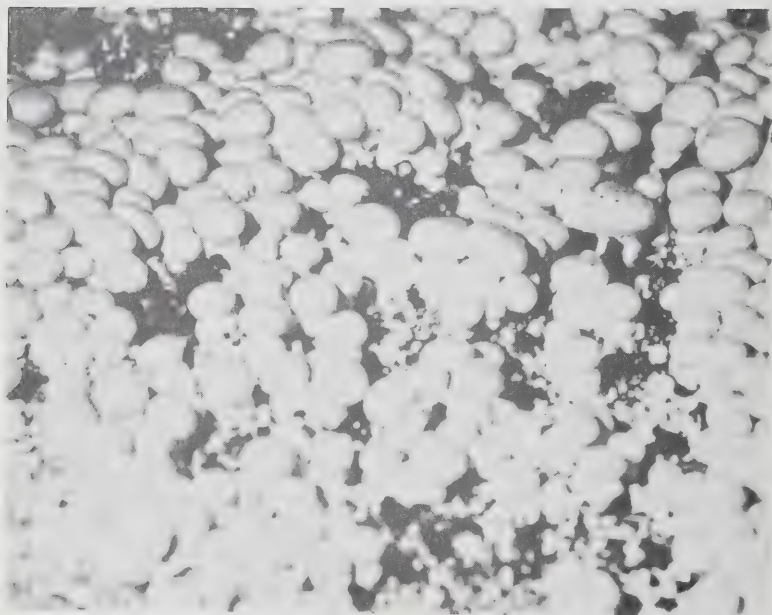


FIG. 1

The first flush of sporophores after about 47 days.

incubated at 53 °C for periods of up to three weeks to provide enrichment conditions allowing the growth of thermophiles. From the third day of incubation the gross cultures were examined with a high power stereoscopic dissecting microscope. Conidia, mycelial fragments or fruiting bodies that could be detected were aseptically transferred to agar plates. These isolation agar plates were inverted, placed in polyethylene bags, and incubated at 53 °C until growth was well established after which the plates were kept at 40 °C. The medium used was Yeast Starch agar (Yp Ss of Emerson, 1941) to which the antibiotic Albamycin (sodium novobiocin) was added at a concentration of 125 mg/l. This antibiotic is thermostable and could be incorporated in the medium before sterilisation. The relatively high level at which the antibacterial agent was used was necessary because thermophilic bacteria were very troublesome at the high incubation temperatures used in these experiments.

In the second method the same procedures were followed as previously described (Eicker, 1972). In brief, the method consists of mixing pulverized pieces of compost directly with molten agar cooled to about 50 °C and pouring plates. In this instance Yeast Glucose agar (Cooney & Emerson, 1964) was used. The same incubation temperature and isolation technique as in the first method were used.

The third method was a modified dilution plate technique devised by Menzies (1957) in which compost suspensions were measured out with a 1 ml dipper which prevented larger and heavier particles settling out and being lost as might have occurred with a pipette. About 5 g fresh weight of compost was accurately weighed out and placed in 250 ml of sterile distilled water in a 500 ml Erlenmeyer flask. This was shaken mechanically for 30 minutes to disperse all materials. A dilution series was prepared from this primary suspension and the degree of the eventual dilution depended on the expected number of viable propagules previously experimentally determined for each stage of composting.

The final dilution factor varied from 1:500 to 1:500 000. One of these dilutions usually gave the optimum of 10–30 colonies per plate. Czapek Dox agar with 0.5 % Difco Yeast extract, rose bengal (1:3 000) and Albamycin (125 mg/l) was used. Ten plates were prepared from each dilution and incubated at 53 °C. The plates were examined after 4 days, the colonies counted, the fungi isolated and the frequency of occurrence (Sewell, 1959) of the different taxonomic entities of thermophilic fungi determined.

The temperature-growth relations of the isolated fungi were determined by growing them in triplicate on Yp Ss and Yeast Glucose agar media. The diameter of the colony was determined daily and visual observations of the degree of sporulation were made after 5 days of incubation at various temperatures.

RESULTS

The results expressed in Tables 2 and 3 and Figures 1 to 6 are based on data obtained from four experiments each comprising the entire 46 day cycle of

mushroom cultivation. More than 400 isolates were obtained using the three isolation methods and these were tested according to criteria for thermophily (Cooney and Emerson, 1964). Ten thermophilic species were identified (Table 3). *Aspergillus fumigatus* was also included amongst these for reasons already given (Eicker, 1972). *Mucor miehei* and *Talaromyces duponti* were isolated by the direct plating technique only while *Malbranchea pulchella* occurred only on pieces of compost that were incubated at 53 °C in a moist chamber. The latter three species were very rare and were isolated once or a very few times only. The remaining 7 thermophiles were much commoner and were readily recovered by all three methods used in this investigation. The temperature-growth relationships of all the isolates showed close agreement with those studied by Cooney and Emerson (1964).

During Phase I the temperature of the stacked composting material reached a peak of about 73 °C on day 4 (Table 2, Figs. 2-7) and then gradually decreased to

TABLE 2
Composting schedule, temperature and pH of the compost.

Days of composting	Composting schedule	Compost temperature	pH	Air temperature
0	Phase I	23,5	8,48	ambient
1		44,0	8,12	
2		62,5	7,97	
3	First turn	67,0	7,94	
4		73,0	7,78	
5		71,0	7,73	
6	Second turn	71,5	7,68	
7		68,0	7,61	
8		61,5	7,40	
9	Third turn	54,5	7,18	
10		49,5	7,10	
11	Phase II	33,5	6,98	40,0
12		41,0		40,0
13		42,5		40,0
14		47,0		40,0
15		54,0	6,93	40,0
16		54,5		40,0
17		53,5		40,0
18		46,0		40,0
19	Cooling down	32,5	7,0	35,0
20	Spawned	27,0		28,0
21		26,5		25,0
22-30	Spawn run	25,0	6,93	25,0
31	Casing applied	18,0	6,63	18,0
32-46	Growth room	18,0	6,62	18,0

below 50 °C by day 10. High temperatures were maintained for 5 to 7 days and the temperature curve shows close agreement with that of grass cutting compost (Webley, 1947) and wheat straw compost (Chang and Hudson, 1967). Phase II temperatures were lower than those of Phase I and reached a maximum of 54.5 °C at day 16. After cooling down at the end of this stage the compost temperatures closely followed the temperature of the air.

TABLE 3

Thermophilic fungi isolated from mushroom compost using three isolation techniques. The temperature range for growth, determined experimentally, is also given.

Species	Temperature range	Methods of isolation		
		Moist chamber	Direct plating	Dilution plate
<i>Aspergillus fumigatus</i> Fresenius ..	15–60 °C	+	+	+
<i>Chaetomium thermophile</i> var. <i>dis-situm</i> Cooney & Emerson	27–58 °C	+	+	+
<i>Humicola grisea</i> var. <i>thermoidea</i> Cooney & Emerson	24–56 °C	+	+	+
<i>Humicola insolens</i> Cooney & Emerson	23–55 °C	+	+	+
<i>Humicola lanuginosa</i> (Griffon & Maublanc) Bunce	30–60 °C	+	+	+
<i>Malbranchea pulchella</i> var. <i>sulfurea</i> (Miehe) Cooney & Emerson ..	27–56 °C	+		
<i>Mucor miehei</i> Cooney & Emerson ..	25–55 °C		+	
<i>Mucor pusillus</i> Lindt	20–55 °C	+	+	+
<i>Talaromyces</i> (<i>Penicillium</i>) <i>duponti</i> (Griffon & Maublanc) emend. Emerson	28–58 °C		+	
<i>Torula thermophila</i> Cooney & Emerson	23–58 °C	+	+	+

The initial pH of the compost was usually above 8. During Phase I the pH dropped to about neutrality where it remained until spawning. After the establishment of the mushroom mycelium there was a gradual but slight further decrease in the pH of about 0.4 units (Table 2).

The occurrence and patterns of succession of the various species of thermophilic fungi encountered in this investigation are discussed separately.

Aspergillus fumigatus Fresenius

The sixty odd isolates of *Aspergillus* fell within the range of *A. fumigatus* (Raper & Fennell, 1965). This organism is extremely plastic and subject to a high mutation rate (Evans, 1971) and this could account for the wide range of

morphological and cultural variations. This characteristic is probably also responsible for the adaptability of *A. fumigatus* and the universal appearance of this fungus on a wide variety of substrates. Throughout the period of observation it was present in the mushroom compost in low numbers with a percentage frequency of occurrence of 15 % or less (Fig. 2). It tended to increase in numbers towards the end of cultivation. Fergus (1964) also noted that it was quite prominent at the end of Phase II of the composting process.

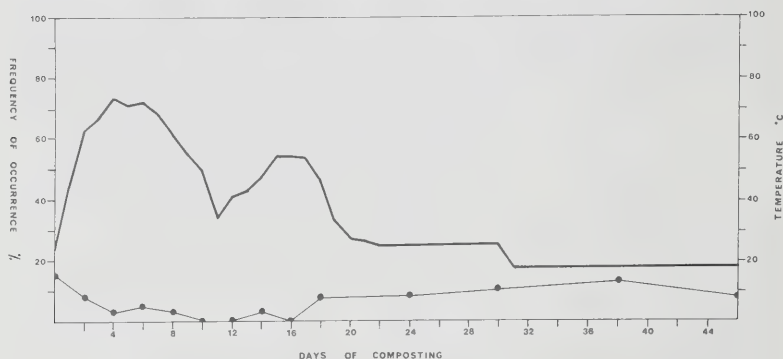


FIG. 2.

Frequency of occurrence of *Aspergillus fumigatus*. In the graph frequency of occurrence the fungal species is represented by ●—●—● while the second curve represents the temperature.

Chaetomium thermophile var. *dissitum* Cooney & Emerson

This fungus formed easily recognisable perithecia on incubated pieces of compost and also on the media used in the other isolation methods. Although Emerson and Cooney's separation of the varieties of the thermophilic *C. thermophile* is rather arbitrary, isolates encountered in this investigation could be accommodated in their var. *dissitum*. This fungus is apparently quite common in composting materials and I have isolated it from several such habitats in this country (Eicker, 1972). Fergus (1964) reported that *C. thermophile* is quite common in mushroom compost during peak heating, but he did not indicate which variety he isolated. The same is also the case with Chang and Hudson (1967) who reported the occurrence of *C. thermophile* on wheat straw compost.

This mould grew very actively during the early stages of composting (Fig. 3), but its numbers decreased during Phase II and had disappeared by the end of the spawn run. Kane and Mullins (1973) have shown that this fungus has a very high cellulolytic ability and its disappearance during spawn run could be attributed to substrate competition with *A. bisporus*.

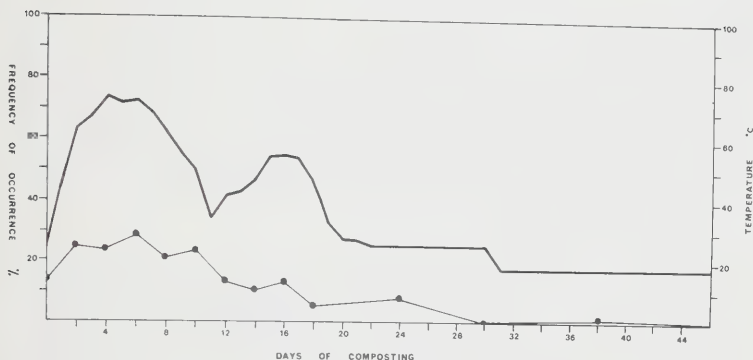


FIG. 3.

Frequency of occurrence of *Chaetomium thermophile* var. *dissitum*. For legend see Fig. 2.

Humicola grisea var. *thermoidea* Cooney & Emerson

The globose to oval, dark brown aleurioconidia which formed on unbranched conidiophores gave this fungus a very characteristic appearance. All isolates agreed well with the species and variety descriptions given by Cooney and Emerson (1964). Occasional intercalary chlamydospores were also produced in substrate hyphae but no phialospores were detected in any of the isolates. The temperature relationships of this thermophile agree well with those given by the original authors. Its occurrence in the compost is high during the thermogenetic stages of composting and its activity correlates very well with the temperature of the substrate (Fig. 4). Two optima are evident in its frequency of occurrence and these correspond with the maximum temperatures recorded during Phase I and

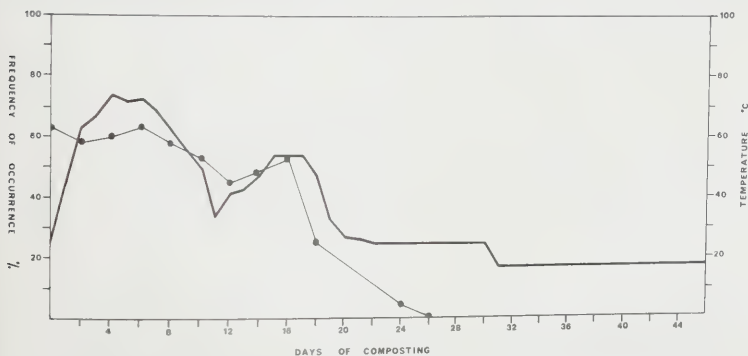


FIG. 4

Frequency of occurrence of *Humicola grisea* var. *thermoidea*. For legend see Fig. 2.

Phase II respectively. At the end of Phase II this fungus can no longer be detected in the substrate.

Fergus (1964) recorded this species from the surface of mushroom compost during peak heating. Hensen (1957) isolated it from horse manure but Chang and Hudson (1967) could not isolate it from wheat straw compost. The possible origin of *H. grisea* might therefore be sought in manure, seeing also that the original habitat of the type material was elephant dung (Cooney & Emerson, 1964).

Humicola insolens Cooney & Emerson

This species, which was also very common in the mushroom compost, could easily be distinguished from the other thermophilic *Humicola* species by its abundant production of light brown, usually globose to oval aleurioconidia, formed mostly in intercalary positions in chains. They also occurred in short lateral chains on lateral aleuriophores. Although this fungus was very abundant during the early stages of composting and showed the same two optima as *H. grisea* var. *thermoidea*, it differed from the latter in that it persisted throughout the entire period of cultivation (Fig. 5). This thermophile was particularly active during Phase II, a fact also noted by Fergus (1964) and Chang and Hudson (1967). Bels-Koning, *et al.* (1962) recorded this fungus, as *Humicola nigrescens* Omvik, towards the end of composting. Hayes (1968) indicated that *H. insolens* and *H. grisea* var. *thermoidea* were the dominant members of the mycoflora at day 3 and subsequently during Phase I and Phase II of the mushroom compost studied by him.

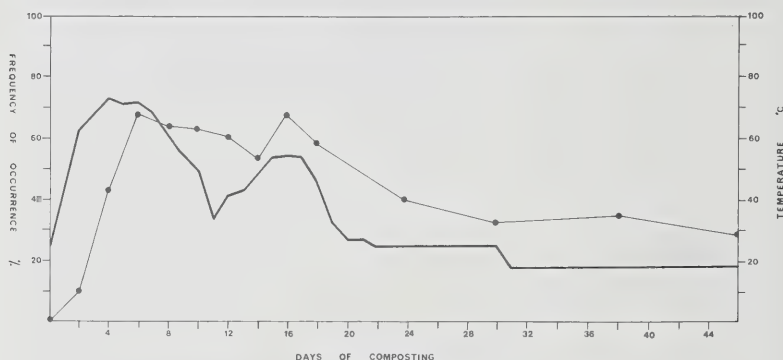


FIG. 5
Frequency of occurrence of *Humicola insolens*. For legend see Fig. 2.

Humicola lanuginosa (Griffen & Maublanc) Bunce

H. lanuginosa was not present at the beginning of the composting cycle. It appeared from the second to the third day and reached a maximum by day 6 of

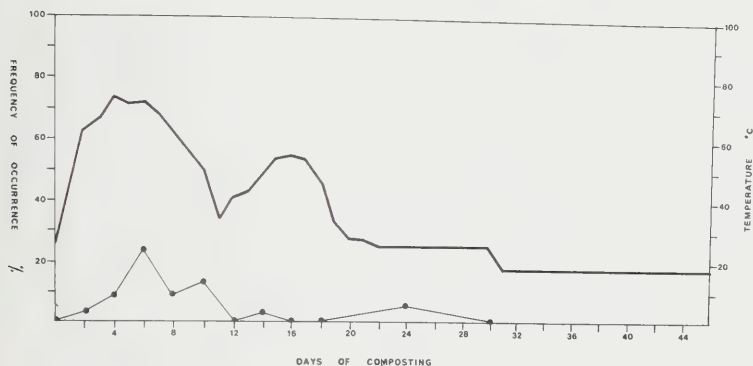


FIG. 6

Frequency of occurrence of *Humicola lanuginosa*. For legend see Fig. 2.

Phase I and then quickly disappeared again (Fig. 6). Fergus (1964) recorded this species only once from the surface of Phase II compost. Chang and Hudson (1967) made records of this fungus before, during and after the heating phase of wheat straw compost. In their investigation of the mycoflora of moist hay undergoing thermogenesis in Dewar flasks, Festenstein *et al.* (1965) found that *H. lanuginosa* was very abundant, producing up to 14 million spores/g dry weight, in the middle layers of the substrate. I have also found this fungus to be extremely common in this country, recording it from 70 % of the substrates which were previously investigated (Eicker, 1972). The taxonomic confusion surrounding *H. lanuginosa* was clarified to some extent by Bunce (1961) and Cooney and Emerson (1964) who considered the genera *Thermomyces*, *Monotospora* and *Acremoniella* to be synonyms for *Humicola*. In his presentation of Dematiaceous Hyphomycetes Ellis (1971), however, follows Apinis (1963) in naming this fungus *Thermomyces lanuginosus* Tsiklinsky.

Malbranchea pulchella var. *sulfurea* (Miehe) Cooney & Emerson

This beautiful thermophilic mould was isolated only three times during this investigation. Colonies developed on pieces of composting material which were collected during the early stages of Phase I and which were incubated at elevated temperature in a moist chamber.

Mucor miehei Cooney & Emerson

Although Sarbhoy (1968) is of the opinion that both the known thermophilic mucors, *M. pusillus* Lindt and *M. miehei* are synonyms, an isolate from early Phase I compost with the direct plating technique, agreed so closely to Cooney and Emerson's fungus that it was designated as *M. miehei*.

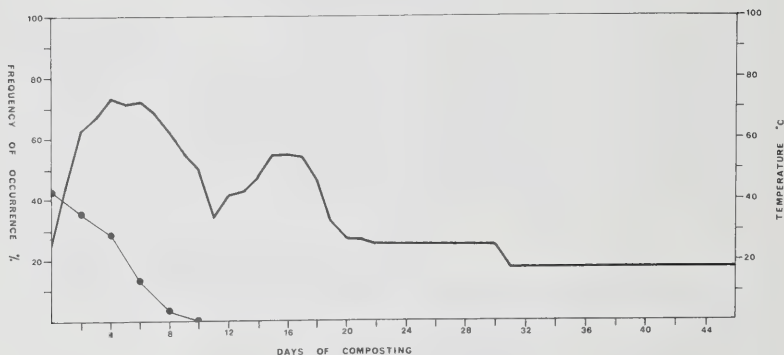


FIG. 7
Frequency of occurrence of *Mucor pusillus*. For legend see Fig. 2.

Mucor pusillus Lindt

This fungus was quite common on substrates used for composting and it had a fairly high frequency of occurrence at the onset of composting. Its numbers rapidly declined and by day 9 it was no longer detectable (Fig. 7). It was recorded by all three methods. As pointed out by Chang and Hudson (1967) the successional pattern of this phycomycetous species is typical of Garrett's (1951) "Saprophytic sugar fungi". Fergus (1964) recorded it only once from a surface sample of Phase II mushroom compost.

Talaromyces (Penicillium) duponti (Griffen & Maublanc) emend Emerson

This thermophilic representative of the Eurotiales was isolated on three occasions by incorporating pieces of compost from late Phase I composting in Yeast Glucose agar and incubating the plates at 53 °C. Chang and Hudson (1967) recorded this fungus from composting wheat straw and Fergus (1964) isolated it from Pennsylvania mushroom compost.

Torula thermophila Cooney & Emerson

Cooney and Emerson (1964), Hayes (1968) and Cailleux (1973) reported the occurrence of this fungus in mushroom compost. In this investigation all three methods employed yielded isolates of this species. Its occurrence was, however, low and confined to Phase II composting with isolation records on days 11, 12 and 15.

DISCUSSION

Composting is a dynamic process with a carefully ordered succession of populations. Hayes (1968), Fordyce (1970) and some other investigators have

indicated that a definite ecological cycle exists and that populations of micro-organisms show a clear and changing pattern. These authors have shown that the early increases and late decreases of temperature during Phase I are paralleled by similar changes in numbers of micro-organisms. This is also true for Phase II which is particularly dominated by thermophilic organisms. Mushroom compost lends itself to the study of microbial ecology, particularly that of thermophiles. The controlled conditions under which the mushroom is grown and the relatively short time required to complete the ecological cycle are favourable factors. There are many practical difficulties in providing a valid qualitative and quantitative estimate of viable micro-organisms. The dilution plate method was adopted in this investigation. Although populations of thermophilic fungi obtained by this method are of doubtful value in absolute terms (Meiklejohn, 1957; Warcup, 1960) it is the most practical and meaningful method for the general study of fungal activity in composts (Hayes, 1968).

With the dilution plate method, in conjunction with two other methods, it was possible to make a comprehensive survey of the thermophilic fungi in mushroom compost and to correlate their occurrence with the temperature changes of the substrate. The activity of these fungi was almost exclusively confined to the heating phases of the compost. The only two fungi which persisted throughout the whole cultivation cycle were *Aspergillus fumigatus* and *Humicola insolens*. It is also clear that many of the thermophiles, such as *Malbranchea pulchella* var. *sulfurea*, *Mucor miehei* and *M. pusillus*, which were present at the onset of composting did not survive long, since they were probably eliminated by the very high temperatures of Phase I.

Dickinson and Pugh (1974) state that well-insulated compost heaps have a considerable heat production which can lead to temperatures in the range of 80–90 °C. Experimental data, however, indicate that at such temperatures the reaction rate of micro-organisms is low. Optimum activity is obtained at temperatures of about 55 °C at which the thermophilic fungi are exerting their maximum influence. The two *Humicola* species, *H. grisia* var. *thermoidea* and *H. insolens*, as well as *Chaetomium thermophile* var. *dissitum* and *Torula thermophila* are the main representatives of this group of active fungi.

The aim of mushroom composting is to produce, largely by the activity of micro-organisms, a substrate which is suitable for the mycelial growth of *Agaricus bisporus*. In the preparation of composts for commercial growing it is essential that all food requirements of the mushroom, which is especially demanding (Smith & Hayes, 1972), are made available by a thorough and carefully controlled composting procedure. This is a demanding and complicated process when variable and bulky materials such as animal manures and other organic substrates are used. Many attempts have been made to accelerate the fermentation by careful preparation of ingredients, mechanical breakdown of vegetable matter (Laborde & Delmas, 1969), carbohydrate supplements (Hayes, 1968) and by inoculating the

compost with suitable thermophilic fungi (Pope, *et al.*, 1962). The application of these and other methods demands a precise knowledge of the nature and activity of the micro-organisms active in composting. It is hoped that this investigation has contributed to this much needed information.

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